Inter- and intraspecific variation in anoxic survival among three bivalve species in intertidal and subtidal areas along the coast of Japan

KATSUMASA YAMADA1*, YASUSHI MIYAMOTO2, TADASHI NAKANO1 & KAZUMARO OKAMURA1

1 Seikai National Fisheries Research Institute, Fisheries Research Agency, 1551–8 Taira-machi, Nagasaki 851–2213, Japan
2 Tottori Prefectural Institute of Public Health and Environmental Science, 526–1 Minamidani, Yurihama-cho, Tottori 682–0704, Japan

Received 22 August 2015; Accepted 8 February 2016 Responsible Editor: Shigeaki Kojima

Abstract: Basal experiments that examine the impact of hypoxia on bivalves provide an explanation for dominant bivalve population maintenance in hypoxic soft-bottom habitats. We conducted laboratory experiments to assess the effect of persistent anoxia on the survival of three bivalve species: Manila clams (Ruditapes philippinarum), ark shells (Anadara kagoshimensis), and Veremolpa micra. Further, we compared the interspecific variation of ark shells among different habitats in the intertidal and subtidal regions of Ariake Bay and Lake Nakaumi, Japan. A marked inter- and intraspecific variation was detected with respect to survival under anoxic conditions. Specifically, the survival of V. micra and ark shells in Lake Nakaumi under persistent anoxia was significantly higher than that of ark shells in Ariake Bay and Manila clams in Tokyo Bay. Further experiments indicated that ark shell survival (LT50) under persistent anoxia with sulfurization was significantly lower than that under anoxia alone. Such inter- and intraspecific variations in anoxia and sulfurization tolerance might reflect the dominance of certain bivalve species in hypoxic regions.

Key words: anoxia, Ariake Bay, hypoxia, Lake Nakaumi, LT50, Ruditapes philippinarum, Anadara (Scapharca) kagoshimensis, sulfurization, Tokyo Bay, Veremolpa micra

Introduction

In shallow coastal areas, persistence of hypoxia for several days to weeks has increased exponentially since the 1960s, with serious implications for the aquatic communities inhabiting these areas (Theede et al. 1969, Shumway et al. 1983, de Zwaan et al. 1995, Diaz & Rosenberg 2008, Vaquer-Sunyer & Duarte 2008, 2010, Conley et al. 2009). This is of particular concern in highly eutrophic areas, such as the inner part of bays and the soft-bottom habitat of estuaries. Many studies have suggested that the occurrence of hypoxia during warmer seasons may lead to a change in the faunal community structure due to the migration of mobile crustaceans and fish, as well as mass mortality of benthic and epibenthic invertebrates, including bivalves, echinoderms, and polychaetes (Shumway et al. 1983, Laudien et al. 2002, Babarro & de Zwaan 2008, Kanaya et al. 2013, 2015, Kanaya 2014).

On the other hand, some infaunal bivalve species can survive better under a wide range of dissolved oxygen (DO) concentrations, and can tolerate extended periods of hypoxia or anoxia (Yurimoto et al. 2008, Miyamoto & Iwanaga 2012, 2016, Suzuki et al. 2012). This is because infaunal bivalves can close their shells to avoid the oxygen depletion in the surrounding water and can increase their anaerobic metabolism to conserve energy with a suppression of aerobic metabolism and survive under hypoxia or anoxia for a limited period. Namely, many infaunal bivalves can tolerate increasing sulfide levels by oxidizing the poisonous compounds into non-toxic compounds, mainly thiosulfate (Isani et al. 1995, Nakamura et al. 1997, Laudien et al. 2002, Babarro & de Zwaan 2008).

Differences of such tolerance against hypoxia and anoxia among bivalve species may be one of the processes that determine bivalve community structure and the dominance of bivalve species at soft-bottom habitats of highly eutrophic, hypoxic regions, such as Tokyo Bay, Lake Nakaumi, and Ariake Bay. Whereas, field observations has suggested that this tolerance alone do not fully explain the

*Corresponding author: Katsumasa Yamada; E-mail, k.yamada@affrc.go.jp
process of the dominance of bivalve species in hypoxic habitats (Yoshino et al. 2007, Como & Magni 2009, Okamura et al. 2010, Miyamoto & Iwanaga 2012, Kanaya et al. 2013, 2015). One of the reasons could be intraspecific differences in response and survival during hypoxia and anoxia under varying hypoxia-periods (from several minutes to several weeks in Tokyo Bay and Ariake Bay, Okamura 2010, Kanaya et al. 2013, 2015, Wakita et al. 2014). A basal experiment that evaluates the inter- and intraspecific variations of hypoxia and anoxia impacts on bivalves might explain the maintenance of dominant bivalve populations in hypoxic regions.

The aim of the present study was to assess the anoxic survival of three infraunal bivalve species, the Manila clam (*Ruditapes philippinarum*), ark shell (*Anadara kagoshimensis*), and *Veremolpa micra*, which are the dominant bivalve species in the intertidal and subtidal muddy sand areas along the coasts of central and southern Japan (Nakamura et al. 1997, Yoshino et al. 2007, Yurimoto et al. 2008, Suzuki et al. 2012, Yamada et al. 2014, Orita et al. 2015). The Manila clam and ark shell are also important fisheries commercial species.

We focused on determining the inter- and intraspecific variation in the anoxic tolerance among species, in an effort to explain the observed differences in the dominant species among regions (Ariake Bay and Lake Nakaumi) and to discuss the processes that maintain the dominant populations in each region. To examine interspecific variation, we focused on the ark shell that commonly dominates the soft-bottom habitat of highly eutrophic, hypoxic regions. Furthermore, the persistence of anoxia progress in this manipulative experiment presumably creates sulfide (H₂S and HS⁻) increasing associated with hypoxia (e.g., Vistisen & Vismann 1997, Yokoyama 2002, Nagasoe et al. 2011, Miyamoto & Iwanaga 2016). This sulfide accumulation under anoxia (dissimilative sulfate reduction) is typically shown in field observations at organic enriched sediment (Sobral & Widdows 1997, Stickle et al. 1989, de Zwaan et al. 1991, 2001a, 2001b, Conley et al. 2009). Therefore, we also assessed the impact of the anoxia but also the sulfide accumulation under anoxia by controlled experimental treatments and discussed negative combined effects of anoxia and sulfide toxicity on bivalve survival.

**Materials and Methods**

**Bivalve collection**

The ark shells and Manila clams were formerly a major fisheries target in the brackish lagoon of Lake Nakaumi, in the inner part of Ariake Bay in western Japan, and in other water bodies in central and southern Japan, Korea, and China. *V. micra* is a small sized bivalve that used to be dominant in subtidal muddy areas along the coasts of southern Japan, such as Wakasa Bay, Amakusa, and Tomoe Bay (Tanaka & Kikuchi 1979, Yokoyama & Hayashi 1980), but recent reports of occurrence and dominance of this species are few (Yoshino et al. 2007, Orita et al. 2015).

The bivalves were collected by SCUBA diving, between July–September 2014, from three regions, i.e., Tokyo Bay, Lake Nakaumi, and Ariake Bay (Fig. 1), but collected bivalve species were different among these three regions. Ark shells were collected from subtidal (33°04′39″N, 130°11′41″E) and intertidal locations (33°05′45″N, 130°22′35″E) at Ariake Bay. Ark shells were extirpated from Lake Nakaumi during the 20th century, probably because of expanding summer hypoxia and sulfide accumulation. Thus, cultured individuals collected from subtidal locations (35°27′59″N, 33°11′29″E) were used for experiments in this study. Ark shells were cultured in pearl nets, hung at a depth of 1.0–1.5 m in Lake Nakaumi, for a period of approximately 1.5 years after larval settlement (cf. Miyamoto & Iwanaga 2012, 2016). *V. micra* were collected only at subtidal areas of Ariake Bay (33°04′39″N, 130°11′41″E).

A uniform size class of Manila clams could not be collected from all three areas, because only young individuals (>20 mm) were found in Lake Nakaumi and Ariake Bay (see also Tsutsumi 2006, Yamada et al. 2014). Furthermore, collected individuals could not be discriminated as source populations for each region (Tsutsumi 2006, Yamada et al. 2014). Therefore, only adult Manila clams were used, collected from intertidal areas at Banzu tidal flat in Tokyo Bay (35°24′N, 139°54′E) (Wakita et al. 2014, Tomiyama et al. 2016). Although we could not compare interspecific variation of anoxia tolerance in Manila clams, previous studies indicated no differences in anoxia tolerance among regions (Sobral & Widdows 1997, Vaquez-Sunyer & Duarte 2010, Nagasoe et al. 2011, Suzuki et al. 2012).

Only adult individuals of each species were used in the experiments. Mean shell length (SL) and wet weight (WT) values were 31.2±1.9, 29.1±3.42, 11.4±0.9 mm, and 6.7±0.7, 7.5±3.50, 0.36±0.09 g for Manila clams, ark shells, and *V. micra*, respectively. Shell length and weight were significantly different among species (one-way ANOVA; SL: F₂, 234=3240, P<0.001 and Tukey test, Manila clam>ark shell>V. micra; WT: F₂, 234=345.4, P<0.001 and Tukey test, ark shell>Manila clam>V. micra), but showed no significant difference among sites and treatments (see below) for each species (two-way ANOVA; SL of ark shell: among collected sites; F₂, 234=1.346, P=0.262, Treatment; F₁, 234=1.704, P=0.193, site×Treatment; F₂, 234=0.013, P=0.987, WT of ark shell: among collected sites; F₂, 234=0.526, P=0.590, Treatment; F₁, 234=0.124, P=0.752, site×Treatment; F₂, 234=0.119, P=0.888, and one-way ANOVA; SL of Manila clam: F₁, 118=0.405, P=0.525, WT of Manila clam: F₁, 118=0.120, P=0.563, SL of V. micra: F₁, 118=0.003 P=0.960, WT of V. micra: F₁, 118=0.004, P=0.952).

In order to eliminate the environmental effect of each location on bivalves (i.e., historical environmental effect), individuals were immediately transported to the laboratory.
Anoxic survival in three bivalve species

After collection and placed in an aquarium (300–500 L) with well-aerated seawater, and acclimated to ambient conditions for a 2-month period. During the acclimation period, media dissolved oxygen (DO) was maintained at 100% by bubbling with air-stones, and the bivalves were fed *Chaetoceros* spp. diatoms (cf. Tomiyama et al. 2016).

Prior to the anoxic exposure experiments, individuals were placed in an aquarium containing well-aerated seawater (200 L) and acclimated to experimental conditions (temperature: 21–28°C, salinity: 32–33), over a period of 5 days, with sufficient bubbling from air-stones and food.

**Anoxic exposure experiment**

Anoxic exposure laboratory experiments were conducted according to previous studies, Stickle et al. (1989), de Zwaan et al. (1995, 2001a, 2002), Babarro & de Zwaan (2008), Nagasoe et al. (2011), and Miyamoto & Iwanaga (2012, 2016). Filtered seawater was made anoxic in a 20 L reservoir by bubbling with nitrogen gas for 2 h. The salinity and pH of the anoxic seawater was approximately 33 and 8.3, respectively. From these reservoirs, seawater was siphoned under continuous flow of nitrogen into 3 and 4 L incubation bottles. After the addition of 10–40 individuals, the bottles were sealed with rubber stoppers and incubated at 28°C. Three to six replicates were carried out for each treatment. The biomass in the bottles was 64.3–105.3 g for Manila clams, 57.5–90.7 g for ark shells, and 6.0–18.6 g for *V. micra*. The wide range of individuals in each bottle (10–40 individuals) based on previous studies listed above. The differences in biomass among 10–40 individuals may slightly affect the results of this experiment (survival of bivalves and changing seawater quality). However, extreme low biomass of *V. micra* might possibly have an effect on the results of this study.

The persistence of anoxic conditions in the experiment bottles presumably created sulfide (H$_2$S and HS$^-$) (e.g., Vistisen & Vismann 1997, Yokoyama 2002, Nagasoe et al. 2011, Miyamoto & Iwanaga 2016). In this study, we define “sulfurization” as progress of sulfide accumulation in seawater under anoxic conditions (dissimilative sulfate reduc-
tion). Therefore, “sulfurization” of this study has a limited sense as creating process of H$_2$S and HS$^-$ under anoxic condition. In order to evaluate the distinct negative effects of anoxia and sulfide accumulation on bivalve survival, we carried out additional experimental treatments as follows: (1) anoxic seawater with sulfurization (natural-treatment), and (2) sulfurization-free anoxic seawater (antibiotic-treatment). The antibiotic chloramphenicol (5 mg L$^{-1}$) was added in the media of the antibiotic treatment every 2–3 days. Chloramphenicol, a broad-spectrum antibiotic, has often been used to inhibit sulfide accumulation in incubation media by preventing the growth of anaerobic bacteria, including Desulfobacteria and Desulfobulbus spp. (de Zwaan et al. 2001a, 2001b, Babarro & de Zwaan 2008, Miyamoto & Iwanaga 2016). Chloramphenicol might also inhibit other bacteria, which attack bivalve muscle (de Zwaan et al. 2001b). Although we could not identify if the antibiotic was inhibiting anaerobic or other bacteria, the studies listed above have confirmed the effectiveness of chloramphenicol in inhibiting sulfide accumulation.

In all treatments, bottles were only opened for a short duration twice daily (every 12 h), to inspect bivalve mortality and to measure water variables (oxygen concentration, oxidation–reduction potential (ORP), and pH) in the incubation bottles. Oxygen concentration was recorded using an LDO-HQ30d (HACH Co., Ltd.), and DO never exceeded 0.3 mg O$_2$ L$^{-1}$. Oxidation reduction potential (mV) and pH were measured with an ORP meter (RM-20P; DKK TOA Co., Ltd.) and pH meter (D-75S; HORIBA Co., Ltd.), respectively, by inserting the sensor probe into the experiment bottle. The pattern of sulfurization and presence/absence of H$_2$S and HS$^-$ in the bottled seawater during the experiments was evaluated by plotting these data into a pH-ORP diagram (Hokari 2012).

When evaluating the presence/absence of H$_2$S and HS$^-$, organic substances derived from the bivalves in the experiment bottles might affect the pH-ORP diagram. This effect may not be observed in the natural-treatment, because sulfate-reducing bacteria can decompose the organic substance, whereas in the antibiotic-treatment, because of the absence of bacteria, the organic substance can affect the pH-ORP diagram. Therefore, evaluating the presence/absence of H$_2$S and HS$^-$ in the antibiotic-treatment might be difficult. In fact, many studies have pointed out a decrease in pH in the antibiotic treatment, because of an increase in organic acid (e.g., lactic acid and propionic acid) (Miyamoto & Iwanaga 2016, see also results in Fig. 3).

No food was added during the anoxic incubations. Mortality was assessed by the failure of constriction after the mantle edge of gaping bivalves were touched (cf. Miyamoto & Iwanaga 2012). Dead bivalves were removed from the incubation bottles.

Statistical Analysis

$LT_{50}$ values (median survival times) were established using the trimmed Spearman–Karber method ($\alpha=10\%$) (Hamilton et al. 1997). Among treatments (effect of the sulfurization under anoxia), species (intraspacific variation), habitats, and regions (interspecific variation) for the ark shell (i.e., among ark shell individuals at intertidal and subtidal areas of Ariake Bay and subtidal areas of Lake Nakaumi) were compared by a one-way, two-way, and nested ANOVA, followed by Tukey’s HSD test for multiple comparisons. If sulfurization during hypoxia affects the survival of bivalve species, $LT_{50}$ would differ significantly among experimental treatments in each bivalve species and/or collected areas (effect of antibiotics). If the impact of anoxia or sulfurization under anoxia demonstrates inter- or intraspific variation, the differences in $LT_{50}$ between experimental treatments would be significantly different. Statistical analyses were conducted using R 3.1.2 (R Development Core Team 2014).

Results

Natural-treatment

Survival time ($LT_{50}$) in anoxic seawater with sulfurization (natural-treatment) was significantly different among bivalve species and collection sites (nested ANOVA; site: $F_{3, 22}=23.94$, $P<0.001$; species (site): $F_{1, 22}=35.36$, $P<0.001$), and also between habitat and region for ark shells (one-way ANOVA; $F_{2, 24}=4.58$, $P=0.021$). LT$_{50}$ values of ark shells in subtidal areas of Lake Nakaumi, and of V. micra in subtidal areas of Ariake Bay, were significantly higher than in other bivalve species (Tukey test, Fig. 2).

Based on the pH-ORP diagram of the natural-treatment (Fig. 3), ORP decrease was more rapid compared to the pH decrease, which could be defined as one of characteristics in sulfurization (compared with pH-ORP diagram in antibiotic-treatment). This is in contrast to the antibiotic-treatment (see below). The pattern of pH-ORP fluctuation (sulfurization) under anoxic seawater also differed among bivalve species (Fig. 3). For example, toxic sulfide (i.e., pH-ORP plots within HS$^-$ or H$_2$S at $LT_{50}$ during the natural-treatment, was not present in V. micra (Fig. 3e), but was present in Manila clams and ark shells (Fig. 3a–d). Thus, with the exception of V. micra, sulfide significantly increased at $LT_{50}$ in all natural-treatments (Fig. 3).

Antibiotic-treatment

$LT_{50}$ between natural- and antibiotic-treatments (i.e., the effect of sulfurization on bivalve survival) were significantly different among bivalve species (i.e., interspecies), as well as among regions (i.e., intraspecies) for the ark shell (i.e., among intertidal, subtidal and collected regions) (Fig. 2). For example, $LT_{50}$ between treatments for Manila clams and V. micra were not significantly different (one-way ANOVA; Manila calm: $F_{1, 5}=1.94$, $P=0.207$; V. micra: $F_{1, 4}=0.025$, $P=0.882$). Conversely, in ark shells, $LT_{50}$ between treatments, and also among collection sites (intertidal and subtidal) and regions (Ariake Bay and Lake
Nakaumi), was significantly different (nested ANOVA; Site: $F_{2, 21} = 293.7$, $P < 0.001$, Antibiotic (Site): $F_{3, 21} = 253.5$, $P < 0.001$).

Based on the pH-ORP diagram for the antibiotic-treatment, pH rapidly decreased, compared to ORP. This was different compared to the natural-treatment and was therefore characterized as slight sulfurization (Fig. 3). For LT$_{50}$, toxic sulfide (pH-ORP plots within HS$^-$ or H$_2$S) was not present in all treatments. The pattern of pH-ORP fluctuation under anoxic seawater with antibiotic was also different between treatments, with differences between species (i.e., interspecies) and among captured areas of ark shells (i.e., intraspecies). For example, at the end of the experiment (LT$_{100}$), toxic sulfide was not present in Manila clams or ark shells from Lake Nakaumi in the antibiotic treatment (Fig. 3a, b). Sulfide in LT$_{50}$ of the antibiotic treatment increased in ark shells from subtidal and intertidal Ariake Bay (Fig. 3a, b), but did not increase in other treatments (ark shells from Lake Nakaumi, Manila clams and V. micra, Fig. 3c, d, e).

**Discussion**

The present study revealed differences in bivalve survival in the presence of anoxia, as well as sulfurization under anoxia, by using a robust and mechanistic laboratory experimental approach. These results are consistent with previous studies that examined survival and hypoxia and anoxia tolerance of Manila clams and ark shells, using a similar approach (Nakamura et al. 1997, Nagasoe et al. 2011, Miyamoto & Iwanaga 2012, 2016, Suzuki et al. 2012). For example, Manila clams ($<25^\circ$C) and ark shells (28°C) survived up to 4–5 days (Nakamura et al. 1997, Nagasoe et al. 2011, Suzuki et al. 2012) and 7 days (Miyamoto & Iwanaga 2012, 2016), respectively, under anoxic conditions. Similarly, in our experiments (summer temperature of 28°C), Manila clams and ark shells survived for a maximum of 3.7 and 6.7 days, respectively, under anoxia (without the addition of antibiotic) (Fig. 2). Especially, ark shells have been reported to survive for 30 days (LT$_{100}$) at 28°C in Lake Nakaumi, under antibiotic anoxia treatments (Miyamoto & Iwanaga 2016). Similarly, in our experiments (28°C) using the same treatment, ark shells from Lake Nakaumi survived a maximum of 29 days (LT$_{100}$).

The present study showed that the effect of anoxia on bivalve survival differed among species (Manila clam, ark shell, and V. micra). LT$_{50}$ (natural-treatment) of ark shells in Lake Nakaumi, and of V. micra in Ariake Bay was significantly higher compared to other bivalve species (Tukey test, Fig. 2). This result indicates that Manila clams
in Tokyo Bay and ark shells in Ariake Bay have a similar tolerance, which is lower than that of ark shells in Lake Nakaumi, and of *V. microa* in Ariake Bay. Especially, this is the first report on the survival of *V. microa* in an anoxic environment, and showed that this species can survive for up to 10 days under anoxic conditions (Fig. 2). Such high tolerance against anoxia may contribute to the recent increase in biomass and species dominance observed in the field, Ariake Bay (Yoshino et al. 2007, Orita et al. 2015). Although regional comparisons of interspecific differences in bivalve survival has been previously evaluated (Vistisen & Vismann 1997, Yokoyama 2002, Nagasoe et al. 2011, Miyamoto & Iwana 2016), we also evaluated the effect of sulfurization under anoxic conditions by manipulate experimental treatments. The *antibiotic-treatment* experiments offer important insights in explaining the processes behind dominant bivalve population maintenance in the soft-bottom habitat of highly eutrophic hypoxic regions. Specifically, the risk of sulfide toxicity under hypoxic conditions (even under short durations of hypoxia, such as in a period of LT50) is important in determining bivalve population maintenance. For example, when sulfide toxicity appeared in the *natural-treatment* of ark shells during the LT50 period (Fig. 3), the mortality of ark shells under combined negative effects of anoxia and sulfurization was significantly higher than under anoxia alone (Fig. 2). The LT50 of ark shells in Lake Nakaumi under anoxic conditions alone was more than triple that of the combined effects of anoxia and sulfurization (Fig. 2). This may be one of the reasons why this species could survive under severe hypoxia observed in the field, such as in Ariake Bay and Lake Nakaumi (Nakamura et al. 1997, Yoshino et al. 2007, Miyamoto & Iwana 2012, 2015, Suzuki et al. 2012). If sulfide toxicity does not increase under progressing anoxia, bivalves could survive for longer periods, even under severe hypoxic conditions. Actually, Miyamoto & Iwana (2012, 2016) and other many reports suggested that benthic mass mortalities during hypoxia may be a consequence of both sulfide toxicity and oxygen deficiency, rather than oxygen deficiency alone, based on physiological perspectives (Shumway et al. 1983, Isani et al. 1995, Vistisen & Vismann 1997, de Zwaan et al. 2001a, 2001b, 2002, Nakamura et al. 1997, Vistisen & Vismann 1997, Miyamoto & Iwana 2016).

The mechanism of such differences in anoxia tolerance between ark shells from Ariake Bay and Lake Nakaumi can be inferred based on differences of the pH-ORP map (Fig. 3). The pattern of decreasing pH and ORP in the antibiotic treatments was notably different between ark shells from Ariake Bay and Lake Nakaumi (Fig. 3a–c). For example, H2S and HS− already arise at LT50 in the *antibiotic-treatment* of ark shells from Ariake Bay, while ark shells from Lake Nakaumi they did not increase until the end of the experiment (LT100), in spite of containing an equal amount of the antibiotic chloramphenicol (Fig. 3a–c). This result indicates that differences in anoxia tolerance between ark shells from Ariake Bay and Lake Nakaumi might be attributed to behavioral differences under anoxic condition. For example, differences in the frequency the shell opens or closes under anoxic condition (frequently open in ark shells from Lake Nakaumi), as well as differences in the amount of sulfate–reducing bacteria (large amount in ark shells from Ariake Bay), might contribute to the observed differences in anoxia tolerance between ark shells from Ariake Bay and Lake Nakaumi.

Conversely, although slight sulfide toxicity may occur in the LT50 period during the *natural-treatment* of Manila clams and *V. microa*, sulfurization (defined as rapid decreasing ORP compared with pH decreasing) certainly occurred (Fig. 3). However, LT50 of Manila clams and *V. microa* did not differ between experimental treatments (Fig. 2). This result supports two possibilities that (1) sulfurization does not affect the survival of these two species, contrary to that of ark shells, and that (2) sulfurization could not advance until effect on survive before sulfide toxicity seriously arose. For example, Manila clams had a lower LT50 compared to the other bivalve species (Fig. 2), suggesting that this species cannot survive for extended periods under the sole effect of anoxia or hypoxia, before sulfide toxicity arose (lower anoxia tolerance than other bivalve species). Actually, it is known that the tolerance of Manila clams to anoxia is notably lower than that of other bivalves, and it has been suggested based on a similar laboratory experiment that sulfide would not be a direct cause of mortality of Manila clams in the natural environment, because of their better tolerance to sulfide compared to hypoxia (Sobral & Widdows 1997, Vaquer-Sunyer & Duarte 2010, Nagasoe et al. 2011). This may be one of the reasons for the high mortality of Manila clams, even during short periods of hypoxia (Tsutsumi 2006, Yamada et al. 2014). On the other hand, *V. microa* had higher LT50 compared to the other bivalve species (Fig. 2), supporting both processes; (1) a lesser effect of sulfurization on survival, during long periods of anoxia, and (2) non-advancing sulfurization before sulfide toxicity reaches critically high levels. However, we could not explicitly identify these processes based on the results from our experiments (Fig. 3). Consequently, the significantly higher tolerance against anoxia demon-
strated by *V. micra*, compared to Manila clams (Fig. 2), may be one of the reasons for the recent dominance of this species in Ariake Bay under severe hypoxia, as suggested by previous studies (Yoshino et al. 2007, Orita et al. 2015).

This study also offers an important indication for the explanation of dominant bivalve species population maintenance in hypoxic regions. LT50 for natural-treatment of ark shells in subtidal areas of Lake Nakaumi was significantly higher compared to ark shells in intertidal and subtidal areas of Ariake Bay (Tukey test, Fig. 2). This result indicates that the ark shells in Lake Nakaumi have higher tolerance than ark shells in Ariake Bay. Furthermore, LT50 for natural- and antibiotic-treatments, and also among collection sites (intertidal and subtidal) and regions (Ariake Bay and Lake Nakaumi), was significantly different, indicating that the effect of sulfurization on the survival of ark shells differed intraspecifically. This is the first report on such a remarkable difference of the effect of anoxia and sulfurization on the survival of the ark shell among regions (i.e., among intertidal, subtidal and collected regions). Ark shells in Ariake Bay displayed a low tolerance against anoxia, where a survival of only ca. 3–4 days (LT100) was documented. Furthermore, in antibiotic anoxia treatments, these individuals could survive for 8–10 days (LT100), which is similar to ark shells in Lake Nakaumi under anoxia (without antibiotic addition). Such low anoxia and sulfide tolerance of ark shells from Ariake Bay may have resulted in a recent mass mortality of this bivalve in Ariake Bay (Okamura et al. 2010, Yoshino et al. 2007).

In conclusion, we detected differences in the degree of anoxia effect on the survival among bivalve species (Manila clam, ark shell and *V. micra*), and interspecifically in ark shells. Furthermore, we also detected that the combined impacts of hypoxia and sulfurization on survival under persistence of anoxia, significantly affect the survival of ark shells, although the survival of Manila clams and *V. micra* suffered less from sulfurization. Our findings highlight the different effects of the duration of hypoxia among bivalve species as well as collected regions, owing to differences in tolerance to hypoxia and sulfurization. This result offers an important insight in explaining the dominance of bivalve species and maintaining their population at the soft-bottom habitat of highly eutrophic hypoxic regions such as Ariake Bay, Lake Nakaumi and Tokyo Bay. However, there still remain for future quantitative studies determining of the effects of sulfide toxicity in bivalves that contain sulfate-reducing bacteria, as a mechanism for survival during anaerobic metabolism (de Zwaan et al. 1991, 1995, 2001a, 2001b, 2002). For example, respiratory pigments differ between bivalve species: Manila clams and *V. micra* have hemocyanin, whereas ark shells have hemoglobin. This might be a possible mechanism to explain our results (lower tolerance of ark shells against sulfide toxicity), because hemoglobin is more responsive to hydrogen sulfide. Such physiological experimental approaches are also required for further studies. This would be also important factor for inter- and intra-specific variation of anoxic survival among bivalve species, needing ingenious experimental system for further understanding (e.g., Miyamoto & Iwanaga 2012, 2016).

**Acknowledgements**

We thank S. Kantoh-Ishizaki, M. Takahashi, H. Hirakiuchi, T. Matoba, A. Nagamoto, K. Yoshino, H. Nakamura, H. Aramaki, Y. Masuda, R. Fukumoto, H. Kanzaki, T. Kurihara, M. Hashimoto, K. Nagasaki, S. Nagasoe, T. Takano, and members of the Inland Water Fisheries and Coastal Fisheries Division in Shimane Prefecture, Saga Prefectural Ariake Fisheries Research and Development Center, Fukuoka Prefectural Fisheries and Marine Technology Research Center, and the Seikai National Fisheries Research Institute (SNFRI), for helping with sample and data collection and experimental setup. This research was supported in part by a contract work with the Ministry of the Environment, Japan, to SNFRI and K. O., and a Grant-in-Aid for Young Scientists (B) (No. 15K18731) to K. Y.

**References**


225–236. (in Japanese with English abstract)


